Gut Microbiome Development and Childhood Undernutrition

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Current human microbiome research is addressing questions first posed by microbiologists more than a century and a half ago; it is doing so with a new and rapidly expanding sets of tools – both experimental and computational.

Introduction

Improving nutritional status is a key component of the United Nations' 2015 Millennium Development Goals [«eradicate extreme poverty and hunger»; «reduce child mortality»; «improve maternal health»] and its 2030 Sustainable Development Goals [«end hunger, achieve food security»]. Historical analyses have emphasized how improved nutrition is a major contributor to economic growth and have underscored the synergism between physiologic improvements due to better nutrition and technical improvements (FOGEL 2004). The rapid expansion of our human population, the existential threat posed by climate change, and the linked need to produce nutritious foods in sustainable ways, highlights a central challenge for the 21st century: build knowledge and technologies needed to make and equitably distribute affordable foods that improve health status. Disappointingly, nutrition has been a neglected area of global health and development, with 88 countries set to miss global nutrition targets set for 2025 (World Health Organization (WHO) Global Nutrition Report, 2020). The COVID-19 pandemic has profoundly disrupted economies, health care delivery, food systems, and many other elements of societies. It has highlighted existing socioeconomic disparities and political complexities and has led to predictions that the global burden of poor nutrition will worsen for sustained periods of time unless new (interdisciplinary) approaches are developed, adopted, and implemented.

Currently, undernutrition is the leading cause of death, worldwide, of children under five years of age. Undernutrition not only increases the risk of death but is also associated with a number of persistent sequelae including stunting, immune dysfunction, and cognitive deficits (BLACK *et al.* 2013). Numerous epidemiologic studies have shown that undernutrition is not due to food

insecurity alone. Moreover, current nutritional interventions, while reducing mortality, have had limited success in overcoming these sequelae (DEWEY – ADU-AFARQUA 2008).

Nutritional status in infants and children is typically defined by measurements of weight and length (height for children); the results are expressed in terms of Z-scores that indicate the extent of deviation from the median value for age-matched members of a multinational reference cohort (WHO Multicentre Growth Reference Study; WHO 2009). Impaired ponderal growth (wasting) is expressed as low weight-for-length/height Z score (WLZ or WHZ). This form of undernutrition affects 45 million children and is classified as «moderate acute malnutrition» (MAM; WLZ between -2 and -3) or «severe acute malnutrition» (SAM; WLZ less than -3). Impaired linear growth (stunting) affects 150 million children and is quantified by length-for-age Z score («moderate» if LAZ is between -2 and -3 and «severe» if below -3). Anthropometry is reasonably straightforward to perform. Nonetheless, it is a «coarse» descriptor of nutritional status and of biological state. This coarseness highlights the need for a more comprehensive definition of the underlying pathophysiologic states of undernourished individuals. The hope is that the process of developing this more comprehensive definition will enable the mechanisms underlying undernutrition to be deciphered, new therapeutic targets to be identified, and the effects of existing as well as new therapeutic modalities to be better assessed.

The WHO recommends exclusive breastfeeding for the first six postnatal months, with continued consumption of breastmilk together with complementary foods through the second postnatal year. WHO has placed complementary foods into eight groups (including breastmilk) and encourages consumption of a minimum of five groups daily (WHO, 2000). Undernutrition frequently becomes evident during the time that complementary foods are administered and has been associated with poor complementary feeding practices (i.e., limited diversity of foods and inadequate representation of micronutrients) (ARIMOND – RUEL 2004; KREBS 2007).

Our intestine is home to tens of trillions of microbes («the microbiota») and their vast collection of genes (the microbiome) – genes that endow us with functional capabilities that we have not had to evolve on our own. Gut microbes belong to all three domains of life on Earth – Bacteria, Archaea, and Eukarya – plus their viruses. Members of Bacteria dominate. Given its role in the biotransformation of the foods we eat, the gut microbiome sits at the interface between food science and nutritional science; it provides us with an opportunity to greatly advance our understanding of our nutritional needs and the link between the foods that we consume and our health status.

The Lab's Encounter with the Human Gut Microbiota

When I was young, I dreamed of being an astronaut – of going to Mars to search for new life. When I was older, I didn't have to travel that far to encounter new lifeforms; a trip a few meters inside was sufficient to begin to see a captivating world of microbes – a «terra incognita».

Our lab's first encounter with the microbiota began with a series of questions related to the continuously renewing epithelium that lines the human gut. We were developmental biologists, interested in understanding how each of the intestine's four principal epithelial cell types establish and maintain their different spatial patterns of gene expression. In other words, how do members of a given epithelial lineage know how to express different sets of gene products in the duodenum compared to the ileum or colon. How are regional differences in gut function established and sustained, and how do they adapt to ecosystem perturbations?

As developmental biologists, we were tempted to turn to the mesenchyme that underlies the epithelium to search for these sources of instructions. However, thinking about postnatal gut development, we turned to the gut lumen. We were fascinated by the thought that the ability of members of the microbiota to establish themselves along the length of the gut might reflect a set of reciprocal signaling events, where the epithelium provides nutritional resources that could be harvested by early microbial colonizers. These early colonizers, in turn, could influence host gene expression and metabolism in ways that would allow them, as well as other organisms, to establish residency in a given region of the gut. We imagined these reciprocal interactions to progress, influencing host and microbial community characteristics in ways that would allow assembly of the community in a dynamically evolving gut habitat. Our underlying assumption was that there was a discernible order to this assembly process in healthy infants and children. Despite the intimating complexity of the system (on the face of things, that there are a seemingly astronomical number of possible pairwise and higher order microbe-microbe and microbe-host interactions that could occur), we hoped to dissect some of these interactions using simplified, representative models of the human gut ecosystem.

We debated about how to create such a system. We started by searching for a human gut bacterial symbiont that satisfied several criteria. It would have to be genetically manipulatable. It would have to be an anaerobe but not too fastidious so that culturing it would not be an overwhelming challenge. It should also exemplify a key functional feature of the human gut microbiota; therefore, we sought a bacterium with a capacity to break down complex

dietary polysaccharides that were not digestible by humans. [Our *H. sapiens* genome possesses only 98 genes encoding glycoside hydrolases and polysaccharide lyases – far too few to process the structurally very complex repertoire of plant glycans that we consume in our diets]. With these thoughts in mind, we traveled 180 miles north from St. Louis to Abigail Salyers's lab at the University of Illinois in Urbana-Champaign and asked her about an organism with a barely pronounceable name that she had been studying - *Bacteroides thetaiotaomicron* (CHENG *et al.* 1992; D'ELIA – SALYERS 1996). She had developed tools for its genetic manipulation. It was an anaerobe that was reasonably straightforward to culture and she had characterized its ability to break down a range of dietary and host polysaccharides *in vitro*. With her characteristic generosity, Abigail opened her lab's freezer (literally and metaphorically) and gave us what we needed to begin.

During the course of our musing about how to create a model system, our attention turned to the field of gnotobiotics; the ability to rear animals, notably mice, under sterile (germ-free) conditions and then to colonize them at a selected time during postnatal life with a given organism or group of organisms. Colonization of germ-free mice can involve direct administration of a microbe, or microbial consortium to an animal by oral gavage, or it can occur «intergenerationally» via transmission from a female mouse to her pups.

Gnotobiotic mouse models seemed like the perfect way to stage, in a highly controlled manner, interactions between a human gut symbiont or symbionts and a mammalian host in specified dietary contexts. Lynn Bry, a courageous and talented MD/PhD student, with the help of Per Falk, a Swedish post-doc in the lab, made arrangements to travel from St. Louis to the gnotobiotic mouse facility at the Karolinska Institute run by Tore Midtvedt, one of the fathers of gnotobiology. She would do experiments there since, at the time, we did not have our own gnotobiotic facility, and when she returned to St. Louis, she would analyze the biospecimens she had obtained.

The results profoundly influenced the course our lab would take. She, Per, and Tore found that germ-free mice initiated a program of expression of fucose-containing polysaccharides on the surfaces of epithelial cells located in the distal small intestine; this induction of fucosylated glycan production occurred during the suckling period. Unlike conventionally raised mice, who acquired their mouse microbiota beginning at birth, this «program» of fucosylated glycan production did *not* generalize in germ-free animals to involve more and more epithelial cells distributed over an ever-increasing area of their distal small intestine. However, colonization of weaned germ-free mice with the fecal microbiota of a conventionally raised mouse (a process known as

«conventionalization») restarted this arrested program of expanding epithelial fucosylated glycan production. Moreover, «mono»-colonization of adult germ-free mice with the wild-type strain of *B. thetaiotaomicron* that we had acquired from Abigail was sufficient to restart this program. Strikingly, experiments using an isogenic mutant strain of *B. thetaiotaoimcron* that was unable to utilize fucose revealed that the induction of host fucosylated glycan expression was dependent upon the ability of the organism to use this carbohydrate as a carbon source (BRY *et al.* 1996).

This finding that a human gut bacterial symbiont could direct its host to manufacture a nutrient source that the microbe could use illustrated how nutrient-sharing relationships are likely key elements underlying establishment and maintenance of mutually beneficial host-microbial relationships in the gut ecosystem. It also pointed to a future where we might be able to deliberately build complexity by colonizing gnotobiotic mice with model human gut communities composed of two, three, four, or many more cultured members of the human gut microbiota. We speculated that these «defined» model human gut communities could provide answers to basic questions about how microbes prioritize, compete for, and/or share available nutrient resources. They could also help us delineate which expressed metabolic activities define their success in different nutrient contexts, in different regions of the gut, and how, in turn, the microbial community members function (collectively) to shape host biology.

With these hopeful thoughts, Lora Hooper, a post-doc in the lab, turned to the then new technology of Affymetrix DNA microarrays to characterize global intestinal transcriptional responses to colonization of germ-free mice with *B. thetaiotaomicron*. The cellular origins of selected responses were subsequently deciphered by laser-capture microdissection of the epithelium. Her results revealed that this organism affected expression of a surprising large range of key functions, including nutrient absorption, gut barrier fortification, xenobiotic metabolism and angiogenesis (HOOPER *et al.*, 2001).

Our desire to understand the mechanisms that allow *B. thetaiotaomicron* to function in the gut led us to sequence its genome. This effort took place in our own lab since the major genome sequencing centers at the time were focused on finishing a draft of the human genome and were limiting their microbial genome sequencing efforts to pathogens. Installing the capacity for microbial genome sequencing in our own lab had a huge impact on how we could and would conduct our studies. For example, we had to acquire the bioinformatic tools needed to annotate the genes in the *B. thetaiotaomicron* genome and predict their functions. This marriage of experimental and computational biology led us, notably, Jian Xu who was a PhD student in the lab, to discover

that *B. thetaioatomicron* VPI-5482 contained a vast repertoire of genes encoding glycoside hydrolases and polysaccharide lyases which were incorporated into multiple polysaccharide utilization loci (PULs) (XU *et al.*, 2003). (To date, 284 glycoside hydrolases, 23 polysaccharide lyases and 96 PULs have been identified in its genome.) Using custom Affymetrix DNA microarrays based on this *B. thetaiotaomicron* genome sequence, Justin Sonnenburg, a post-doc in the lab, was able to explore how its PULs are regulated *in vivo* (in gnotobiotic mice) and *in vitro*, and how it adeptly uses its PULs to adaptively forage on dietary versus host glycans depending upon the availability of polysaccharides in the diet (SONNENBURG et al., 2005). Follow-up studies by Eric Martens and subsequently other members of the lab, including Andy Goodman, Jeremiah Faith, Nate McNulty, Meng Wu, Michael Patnode and Darryl Wesener, focused on how PULs had evolved across other human gut *Bacteroides* species and strains, how the genes comprising PULs contribute to fitness in different community and dietary contexts, and how the products of carbohydrate metabolism are shared among members of the gut community.

This marriage of gnotobiotics, functional and comparative genomics greatly expanded our appreciation of how a human gut symbiont could influence many aspects of host biology and spawned a next phase of our research that involved colonizing germ-free mice, and later other host species, including germ-free zebrafish and germ-free piglets, with defined microbial communities of increasing complexity and diversity, composed of cultured, sequenced members of the human gut microbiota. Encouraged by the wealth of new information about the functional capabilities of cultured human gut symbionts that were coming from our studies, Ruth Ley, a post-doc in the lab and I, together with several colleagues, submitted a white paper in 2005 to NIH's National Human Genome Research Institute proposing a human microbiome initiative that would deliver deep draft whole genome sequences for 100 organisms representing major phylogenetic lineages in the human gut microbiota (https://www.iccueducation.org.uk/uploads/2/3/1/0/23109338/white_paper.pdf). We argued that these characterized microbial genomes would herald another phase of the «human» genome sequencing project, provide a key reference for subsequent human microbiome projects, and serve as a model for future initiatives seeking to characterize other, extra-intestinal microbial communities. The white paper was approved.

Childhood Undernutrition and Gut Microbiome Development

To translate our findings from these preclinical models to humans, we began a journey designed to explore the role of the gut microbiome in malnutrition, including one of its manifestations – childhood undernutrition. This journey

began with a hypothesis, namely, that there is a definable normal program of gut community development which is perturbed in children with undernutrition, and that this perturbation is a contributing cause rather than «simply» an effect of undernutrition. Corollaries to this hypothesis are that healthy growth of infants and children is linked to healthy development of their gut community and that «repairing» the microbiome would offer an opportunity to link community components and their functions to various host systems that regulate growth.

We envisioned that testing this hypothesis would demand a series of steps. First, we would have to develop the means to define «normal» gut microbial community development in a given population of healthy infants and children and then determine if that definition generalized to other populations representing different geographic locales and anthropologic features. Next, we would need to apply this definition of normal to identify and quantify disruptions in community assembly and determine whether the degree of disruption is significantly correlated with the degree of undernutrition (growth faltering). If these correlations were observed, we would proceed to test, initially in representative preclinical models, whether disrupted microbial community development is a cause or an effect of undernutrition. If we «passed» this test, we would proceed to use these preclinical models to identify components of the developing gut community that could serve as targets for therapeutic interventions. If and when such targets were identified, we would use preclinical models that harbor gut microbial community components from the human population we wished to treat to develop therapeutic candidates. We would then be in a position to determine the efficacy and safety of lead therapeutic candidates by «returning» to the very human population(s) where the microbial community perturbations were initially characterized and whose microbial communities had been employed to create the preclinical models. We reasoned that randomized, controlled clinical trials of microbiota-directed therapeutic candidates should include a comprehensive definition of the biological state of participants prior to, during, and following treatment so that we could begin to connect, mechanistically, community repair to alterations in the operations of host systems and subsystems. We also envisioned that if proof-of-concept was established in randomized controlled clinical studies, we could reenact the study, in our preclinical models, using pre-treatment microbiota from participants (or cultured members of their gut communities); these «reverse translation» studies might allow us to further identify the molecular mediators that link community repair to alterations in host biology (e.g., metabolic regulation, musculoskeletal development, the operations of the innate and adaptive arms of the immune system, and neurodevelopment).

In addition to these considerations, we knew that an essential component of this multi-step translational medicine «journey» would be to focus on therapeutic approaches that would be affordable, culturally acceptable, and scalable. Moreover, we would need to determine the extent to which therapeutic targets are unique to a given population or shared more broadly. The motivation to obtain an answer to this latter question is that an «efficiency of scale» would be achieved with an intervention that had broad cultural acceptability and where efficacy generalized to undernourished children living in different areas of the world.

Defining «normal» and deviations from normal – Acquisition of an infant's gut microbial community begins at birth. Therefore, to define «normal», our long standing collaborators at the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b), led by Tahmeed Ahmed, conducted a birth cohort study in which fecal samples were serially collected from postnatal months 1 through 60. Study participants lived in an urban slum in one of the districts of Dhaka Bangladesh. Focusing on samples serially collected from cohort members who exhibited consistently normal anthropometry during this period, we used culture-independent methods to define the bacterial composition of their developing microbiota. This enumeration effort entailed sequencing PCR amplicons generated from 16S rRNA genes present in their gut communities; this gene is a phylogenetic «barcode» and variations in its sequence allow classification of bacterial taxa. By applying various feature selection methods to this large dataset (initially machine learning algorithms and later methods developed by the field of econophysics), two talented members of our group, Sathish Subramanian and Arjun Raman, identified a sparse group of «age-discriminatory» bacterial taxa whose changing patterns of representation and co-variation operationally defined a program of gut microbial community assembly shared between these healthy children. This program appeared to be largely completed by the end of their second postnatal year (SUBRAMANIAN *et al.*, 2014; RAMAN *et al.*, 2019). Follow-up studies, conducted in several low- and middle-income countries, disclosed that this sparse group of organisms (which Arjun termed an «ecogroup») was also represented in healthy members of other birth cohorts.

Together with our icddr,b colleagues, we found that children with moderate or severe acute malnutrition had perturbed community development, as judged by aberrant representation of these taxa as well as microbial genes comprising various metabolic pathways; their microbial communities had configurations resembling those of chronologically younger children. The degree of perturbation appeared to be correlated with the severity of undernutrition (e.g., worse in children with severe compared to moderate acute malnutrition). Moreover, this «microbiota immaturity» was incompletely repaired by

standard nutritional interventions (SUBRAMANIAN *et al.*, 2014; GEHRIG *et al.*, 2019) – interventions that were not designed based on knowledge or consideration of gut microbial community development. In other words, these children were left with a persistent developmental abnormality affecting one of their «organs», albeit a microbial one.

A test of causality. To determine whether perturbed gut microbial community development is a cause rather than simply an effect of undernutrition, members of the lab colonized separate groups of gnotobiotic mice with fecal samples collected from healthy children and from their chronologically aged-matched counterparts with acute malnutrition. All mice were given the same diet – one representative of that consumed by the children whose microbiota had been sampled. The results revealed that communities from undernourished children caused impaired weight gain, altered bone growth, and produced immune and metabolic abnormalities (SMITH *et al.*, 2013; KAU *et al.*, 2015; BLANTON *et al.*, 2016; GHERIG *et al.*, 2019). Application of machine learning methods to the transplanted communities revealed «growth-discriminatory» bacterial taxa, defined as organisms whose abundances correlated with the growth phenotypes of recipient gnotobiotic animals. Co-administration of a consortium of these (cultured) growth-discriminatory taxa with intact microbiota from an undernourished donor produced changes in lipid and amino acid metabolism and augmented lean body mass in recipient gnotobiotic mice compared to animals that received the undernourished donor microbiota alone (BLANTON *et al.*, 2016). Intriguingly, a number of these growth-discriminatory taxa were under-represented in the fecal microbiota of children with moderate and severe acute malnutrition during the period of complementary feeding. These organisms became our therapeutic targets.

Development of microbiota-directed complementary foods – Our therapeutic goal was to develop a way of nutritionally resuscitating children that would not only involve provision of additional calories and micronutrients, but also result in restoration of age-appropriate co-development of the gut microbiota and host. However, this strategy raises a number of intriguing questions about the «malleability» of this co-developmental process. How «fixed» is the functional configuration of a community that has co-existed with undernourished children for different periods of time? How fixed is the host physiologic state or states associated with undernutrition/impaired growth? To what extent does the «malleability» of this state or states relate to the severity of the microbial community disruption and the chronologic age of the child? Malleability can be viewed as the capacity of growth-promoting taxa (and microbiome-encoded metabolic and signaling pathways) represented in the perturbed microbial communities of affected individuals to re-establish their

health-promoting functions during and following completion of community repair. Given the complexity and dynamism of microbe-microbe and microbe-host interactions, we also wondered how the rate and degree of repair («catch-up development») of a perturbed community would relate to the degree of restoration of healthy growth. In addition, we had little information about «biogeography»; i.e., what regions of the intestine do growth-discriminatory organisms occupy in healthy children versus those with «undernutrition», and does their location reflect different ways that normal versus perturbed communities can affect growth?

With these questions and thoughts in mind, we hypothesized that certain complementary foods might contain ingredients that affected the fitness and expressed beneficial activities of age/growth-discriminatory strains inappropriately represented in the developing gut communities of 12- to 18-month-old children with moderate acute malnutrition (MAM). We focused on MAM, this age range, and this approach for several reasons. First, there are no generally accepted recommendations for treating MAM. Second, the target age range was one where the disease is commonly diagnosed. Third, different complementary foods might be combined to create culturally acceptable, affordable, and efficacious microbiota-directed complementary food formulations (MDCFs) that could, in principle, be readily produced at scale.

Our preclinical screens of locally available complementary food ingredients were first conducted in gnotobiotic mice colonized with a consortium of genome-sequenced, age- and growth-discriminatory bacterial strains. These strains had been cultured from fecal samples collected from Bangladeshi children residing in the very communities where MDCFs would be first tested. Ingredients that promoted the fitness of strains deficient in the microbial communities of children with MAM were advanced by members of the lab, notably Jeanette Gehrig, Sid Venkatesh, and Hao-wei Chang, to a series of secondary tests in gnotobiotic mice colonized with intact fecal microbial communities sampled from undernourished children who had been treated with standard nutritional interventions but, nonetheless, were left with persistent microbiota immaturity. Lead formulations that (i) repaired the transplanted microbial communities, (ii) had favorable effects on various facets of growth, and (iii) were comprised of ingredients that had satisfactory organoleptic properties were subjected to a tertiary test of efficacy in just weaned gnotobiotic piglets that had been colonized with members of the microbial communities of the target human population. We selected piglets as a second host species because their physiologic and metabolic properties are closer to those of humans than those of mice. We also reasoned that their growth potential in early postnatal life is so great that we could readily ascertain the effects of MDCF prototypes in these animals. We invested

considerable time and energy to conduct this tertiary test in gnotobiotic piglets because we wanted to adopt a very cautious approach in our translational efforts: this embrace of the precautionary principle was based on the possibility that our interventions could have substantial effects on child development. Three prototype formulations emerging from this preclinical pipeline were subsequently tested in a 4-week pilot study in 12- to 18-month-old Bangladeshi children with MAM where the outcome measures were microbiota repair (as judged by the representation of ecogroup taxa in their fecal samples), and changes in levels of plasma protein biomarkers and mediators of different aspects of healthy growth (GEHRIG *et al.*, 2019; RAMAN *et al.*, 2019).

A follow-up, longer (3-month), more highly powered, randomized controlled trial (RCT) was subsequently performed by Tahmeed Ahmed and his group in 12- to 18-month-old Bangladeshi children with MAM. The diets of study participants were supplemented with a lead formulation known as MDCF-2; this formulation was identified in the pilot study as producing more complete microbiota repair than the other MDCFs tested. In the 3 month-long RTC, MDCF-2 produced superior rates of ponderal growth (improvements in WLZ) compared to a current ready-to-use supplementary food (RUSF) – even though the RUSF had 15% higher energy density (CHEN *et al.*, 2021).

To explore the underlying mechanisms, Matt Hibberd, a post-doc in the lab, and Robert Chen, a MD/PhD student, performed bacterial 16S rRNA gene amplicon sequencing on fecal samples serially collected prior to, during, and after MDCF-2 or RUSF treatment. Using linear mixed effects models, they identified taxa (amplicon sequence variants or ASVs) whose abundances were significantly associated with improvement in WLZ. Twenty-one bacterial taxa were significantly positively associated with WLZ and two were significantly negatively associated with WLZ (CHEN *et al.*, 2021). A number of these taxa, including *Prevotella copri,* are associated with the weaning phase microbiota of healthy Bangladeshi children, and depleted in chronologically age-matched Bangladeshi children with acute malnutrition (SUBRAMANIAN *et al.*, 2014). Several other WLZ-associated taxa had been identified as «growth-discriminatory» in our gnotobiotic mouse studies and were among the bacterial targets used in our screens of complementary food ingredients that led to development of MDCF-2 (GEHRIG *et al.*, 2019). Importantly, compared to RUSF, MDCF-2 produced a significantly greater increase in taxa that were positively associated with WLZ.

To delve further into mechanism, Robert Chen used an aptamer-based proteomics platform to quantify the effects of the two treatments on levels of nearly 5000 plasma proteins; these proteins included numerous biomarkers

and mediators of various facets of host physiologic processes. Analyzing all participants in study, and using linear mixed effects models, we identified a group of ~70 plasma proteins whose levels were positively associated WLZ. They included proteins involved in musculoskeletal and central nervous system development. Others, including activators of immunoinflammatory responses, were negatively associated with WLZ. As in the case of the ASVs, when compared to RUSF, MDCF-2 produced significantly greater increases in levels of proteins positively associated with WLZ, and significantly greater reductions in levels of those negatively associated with WLZ (CHEN *et al.*, 2021).

Identifying ASVs allowed us to assign names (membership in different taxonomic groups) to treatment-responsive/WLZ-associated microbial community members. However, we needed to go well beyond this level of description of microbial community response; namely, to identify functional features (e.g., metabolic pathways) that are significantly enriched in the genomes of these organisms. We also wanted to move beyond DNA-level descriptions to an assessment of the expression of these features. Fortunately, reductions in the cost of shotgun sequencing of microbial community DNA, coupled with improvements in the algorithms used to assemble the reads into MAGs (metagenome-assembled genomes), has opened the door to these types of analyses. Therefore, members of the lab (Matt Hibberd and Dan Webber) have used the fecal samples collected prior to, during, and at the end of treatment from each study participant to (i) assemble MAGs, *de novo*, that were present in this cohort, (ii) determine how the abundances of these MAGs (bacterial strains) were affected by treatment, and (iii) perform *in silico* reconstructions of metabolic pathways encoded by these MAGs. Finding that pathways involved in carbohydrate utilization were the most significantly enriched in treatment-responsive and WLZ-associated MAGs, they proceeded to use microbial RNA sequencing to identify which of these carbohydrate utilization pathways are differentially expressed in MDCF- compared to RUSF-treated children, identify the MAGs that are the principal contributors to these differentially expressed pathways, and integrate these transcriptional analyses with mass spectrometry-based analyses of carbohydrate structures present in MDCF-2, RUSF, and the collected fecal samples. The results have provided insights about which WLZ-associated bacterial strains are principally responsible for metabolism of MDCF-2.

These findings set the stage for ‘reverse translation’ experiments. These types of experiments involve gnotobiotic mice colonized with pre-treatment ‘unrepaired’ and post-treatment ‘repaired’ communities from clinical trial participants, or with consortia of age-/WLZ-associated organisms, cultured from the target population and selected based on the degree to which their

genome content resembles those of the corresponding MAGs. Mice colonized with systematically manipulated consortia of the cultured organisms (e.g., 'leave-one-out' experiments) enable more detailed analyses of the principal consumers of MDCF components. They also allow tests to be performed of whether these, or other, community members are the principal drivers of various growth phenotypes. In addition, they serve as an experimental platform for conducting mass spectrometry and functional genomic assessments of the metabolic and signaling pathways that mediate the effects of these organisms on various host cell lineages within and outside of the gut. The resulting deeper understanding structure/activity relationships for MDCF formulations will help guide decisions about dosing, and provide a path forward for generating biosimilar, shelf stable MDCFs with acceptable organoleptic properties where the ingredients may come from various sustainable foods sources that can be produced at affordable prices.

Looking Ahead

Childhood undernutrition reflects the impact of poverty and social inequality over many generations. It has many contributing biological factors. Evidence is accumulating that perturbed development of the microbiome during the critical first two years of life is one such factor. This microbial perspective about disease pathogenesis, treatment, and prevention invites many thoughts about what needs to be done next.

Biogeography - Much of the work to date in the field of gut microbiome research has focused on analyses of fecal samples due to their ease of collection. In contrast, the small intestinal microbiota has remained an underexplored 'wilderness' due to the difficulty in obtaining samples. This limitation underscores the need to invest in developing less invasive sampling techniques where sampling can be performed repeatedly and safely over time (Tang et al., 2020). The hope is that this effort will enable more information to be gleaned about the mechanisms by which members of the microbial community, positioned in different regions of the intestine, contribute to host growth and metabolism. A corollary is that this knowledge would facilitate the development of therapeutics with more comprehensive biogeographical coverage and hence more complete repair of community and host co-development.

Generalizability - The translational medicine approach described above provides a roadmap for testing the generalizability of the effects of MDCFs across different study populations, and the impact of this type of intervention at different ages in children with different degrees of disease severity. To simplify global implementation, policies for nutritional supplementation have traditionally been applied across a broad range of ages encompassing the

entire period of complementary feeding (e.g., 6-24 months). From the perspective of microbial community development, this represents a potentially wide range of evolving therapeutic targets, which may require a sequence of different MDCFs applied in succession.

Dosing and complementary feeding practices - As more information is obtained about the structure/activity relationships of therapeutic foods (i.e., the nature of their bioactive ingredients), attention will need to be paid to defining dose-response relationships as a function of age and disease severity (including co-morbidities), as well as determining the duration of administration of a given sequence of MDCFs for sustained growth promotion. Deeper knowledge of these structure/activity relationships may enable an evolution in policies for treating undernourished children beyond current recommendations, which are based largely on energy density and macro/micronutrient content. One hoped for return on investments that seek additional food staples that possess similar or superior bioactivity to already tested MDCF ingredients is a knowledge base that will lead to new guidelines for complementary feeding practices – guidelines informed by knowledge of how to better promote healthy development of a gut microbial community.

Significance of strain-level variation. The work with MDCFs has underscored the importance of understanding the significance of strain-level diversity in the gut community, with different MAGs belonging to the same «species» having different genomic features that (i) affect their capacity to utilize and respond to bioactive components present in these types of therapeutic foods, and (ii) determine their relationship to host growth responses. As noted above, more work is needed to ascertain the degree to which repair of perturbations in gut microbial community-host co-development at different stages of postnatal life requires targeting different organisms/metabolic pathways. In addition, the efficacy of MDCFs may be very limited if the gut ecosystem is so perturbed that microbial therapeutic targets are absent or severely depleted. Under these circumstances, community restoration may require a form of regenerative medicine where these organisms are administered as a next generation probiotic formulation, or a probiotic followed by an MDCF, to induce and sustain a growth-promoting gut microbiome. Culturing and selecting strains based on the degree to which they approximate metabolic pathway and other features documented in growth-associated MAGs would represent one facet of this effort. An additional consideration in selecting strains as candidate probiotics is whether they are from hosts whose environmental exposures resemble those experienced by the target population of undernourished children; these strains may yield higher engraftment efficiencies, and thus have more impactful health benefits. Moreover, multi-strain consortia should be used to optimize the chance of engraftment in the

gut microbial communities of recipients representing different populations, thus mitigating the need to generate multiple, distinct, «geo-adapted» individual strain formulations (BARRATT *et al.*, 2022a,b)?

Intergenerational transmission of the microbiome. The principal source of microbes in the infant gut is the maternal microbiota. As such, a comprehensive understanding of host-microbiota co-development requires a deep and sustained investment in the characterizing of the microbial ecology/dynamics of the maternal-child «dyad». However, comparing the microbial communities of healthy and undernourished women and the intergenerational transmission of these communities requires prolonged, complex, adequately powered longitudinal studies. These observational studies need to be linked to preclinical intergenerational transmission models representative of the human cohorts being characterized so that disease mechanisms and therapeutic targets can be identified. Critically, in populations where the burden of maternal and child undernutrition is great, focusing on those who have avoided undernutrition (i.e., have exhibited «positive deviance») is a first and necessary step in defining what is normal (DAS *et al.*, 2022).

Surmounting the challenges and conducting type of studies described above provides an opportunity to improve growth and developmental outcomes. One hoped-for fruit of these efforts will be a new generation of cost-effective point-of-care diagnostics for defining wellness, and for designing more effective preventive measures for populations deemed at risk for developing undernutrition. My hope for all of us is that we can become better stewards of our children's precious microbial resources and in this and many other ways help them lead healthy productive lives so that they can realize their full potential.

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